Figure 1 A

SEQ ID NO: 1

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ITH

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DRSLLEYVNHSNTT"

Figure 1 B

SEQ ID NO: 2

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SEQ ID NO: 3 Figure C

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SEQ ID NO. 4

Figure 1D

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Figure 1

TRAC1 genomic region:

SEQ ID NO:5

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Figure [redacted] cont'd

SEQ NO:5
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Figure 1 E cont'd

SEQ NO:5 cont'd

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Figure 1 F

SEQ ID NO: 6

Mouse TRAC1 cDNA sequence:

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SEQ ID NO: 7

Mouse TRAC1 protein (3rd frame)

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ELDEDCLLDHCIIHHRSERRPVFCPLCHSRPDESPSTFNGSLIRHLQVSH

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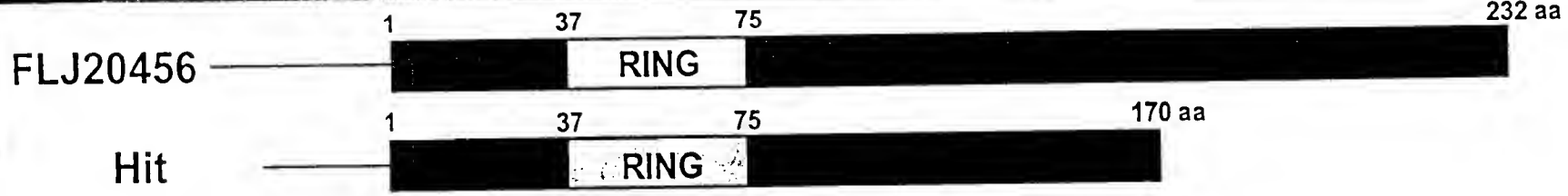
FLJ20456 Hit Inhibited anti-TCR Induced CD69 Expression in Jurkat Cells



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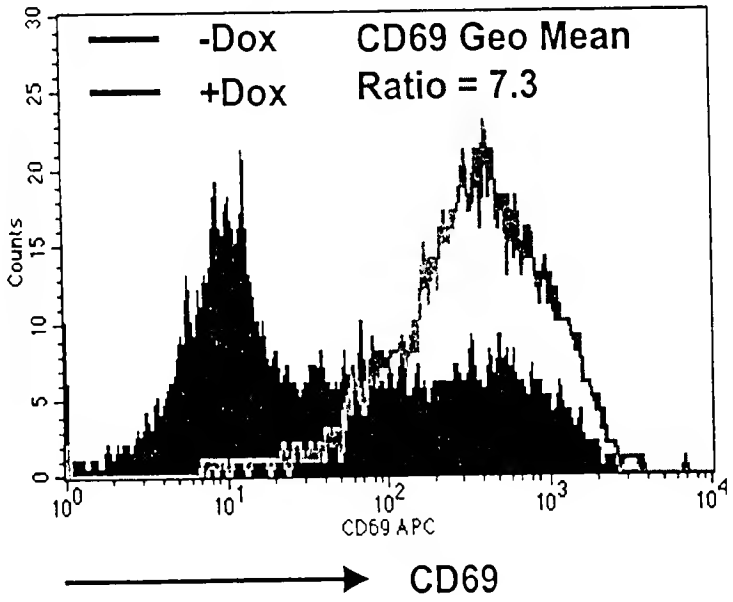

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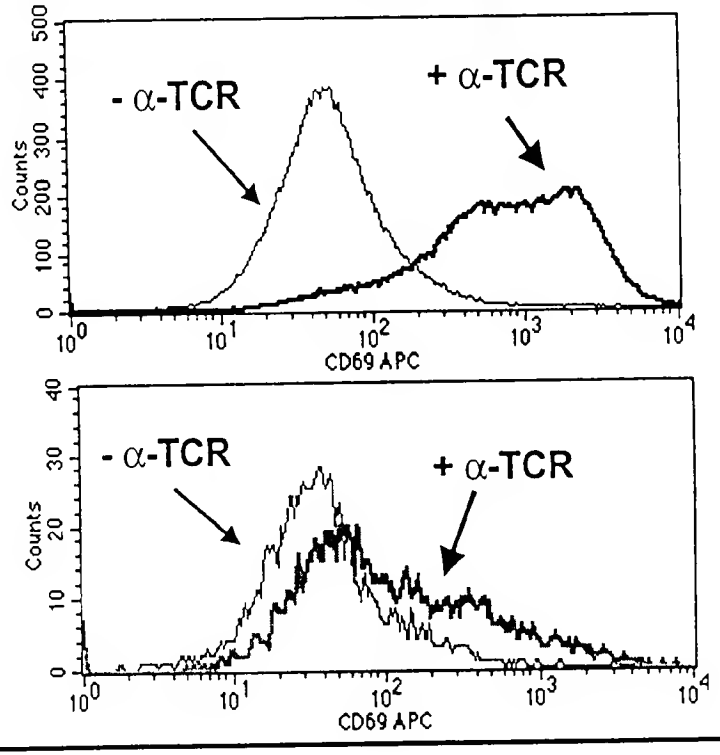
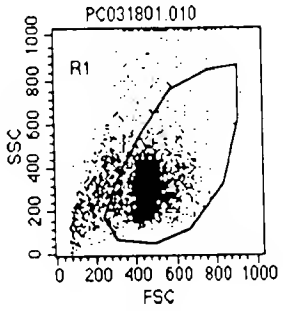


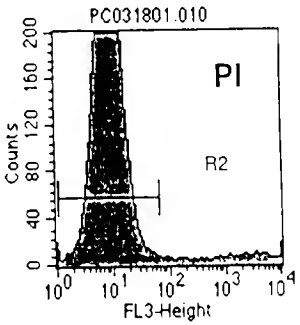
Figure 2

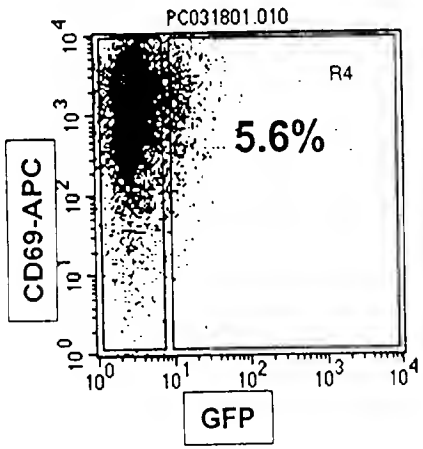
Full Length FLJ20456 Does Not Inhibit CD69 Upregulation in Jurkat Cells

- Pfu PCR product amplified from a capped human brain cDNA library.
- One N to S polymorphism with FLJ20456 NM_017831.1 at amino acid 186, present in EST database.

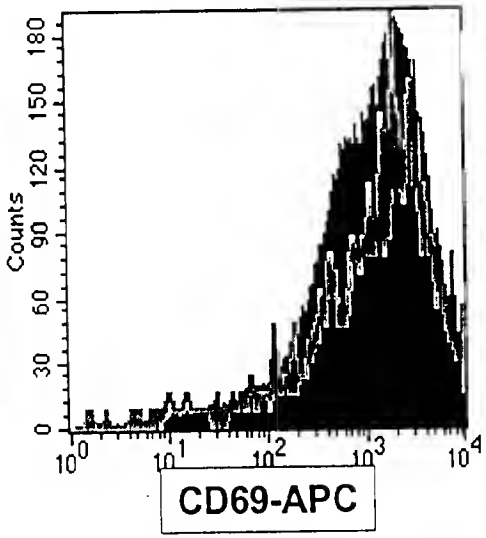
JurkatN 32H







300ng/ml C305



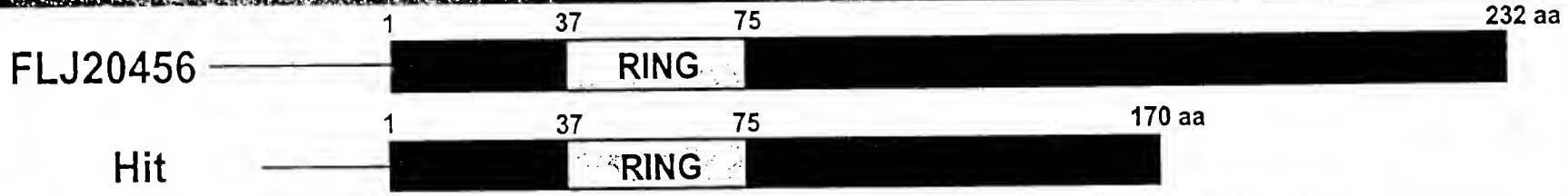
GFP- 1070.5

GFP+ 1219.9

Ratio = 0.88

Figure

FLJ20456 Hit Specifically Inhibited T Cell Activation but not B Cell Activation

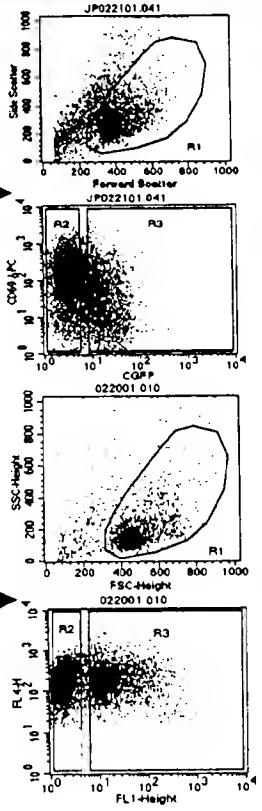


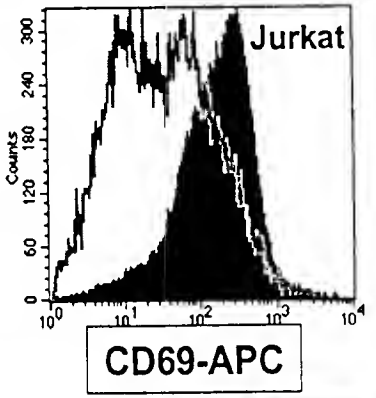
Transfect Phoenix cells with TIG-FLJ20456 hit

Viral supernatant

Infect Jurkat and Assay

Infect BJAB and Assay



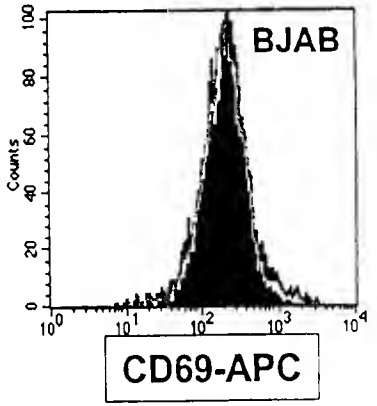


Stimulation: 300ng/ml C305

GFP- 141.2

GFP+ 28.8

Ratio = 4.9



Stimulation: 300ng/ml α-IgM

GFP- 183.1

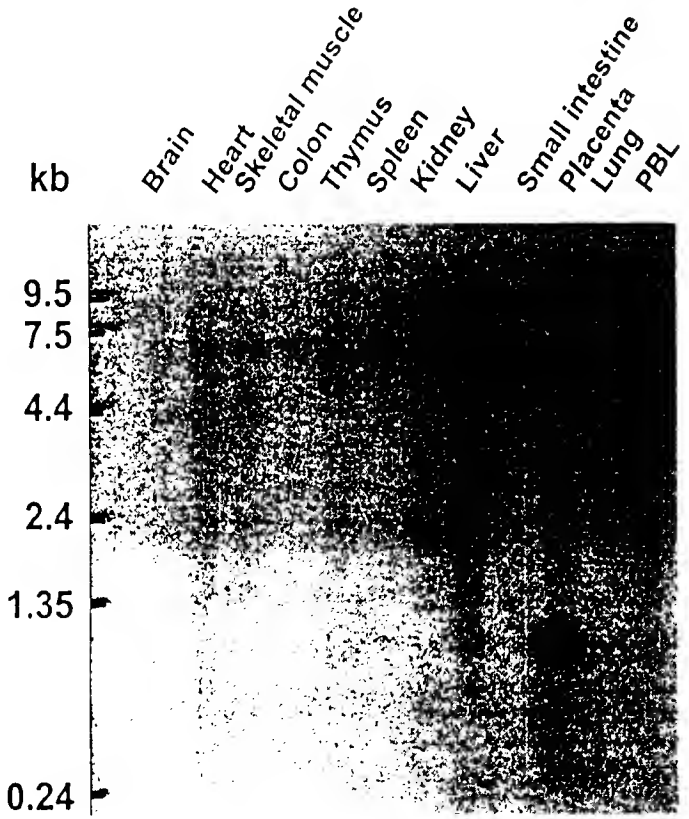
GFP+ 183.3

Ratio = 1.0

Figure 4

FLJ20456 Is Strongly Expressed in Lymphoid and Hematopoietic Organs

Probe: FLJ20456 Hit

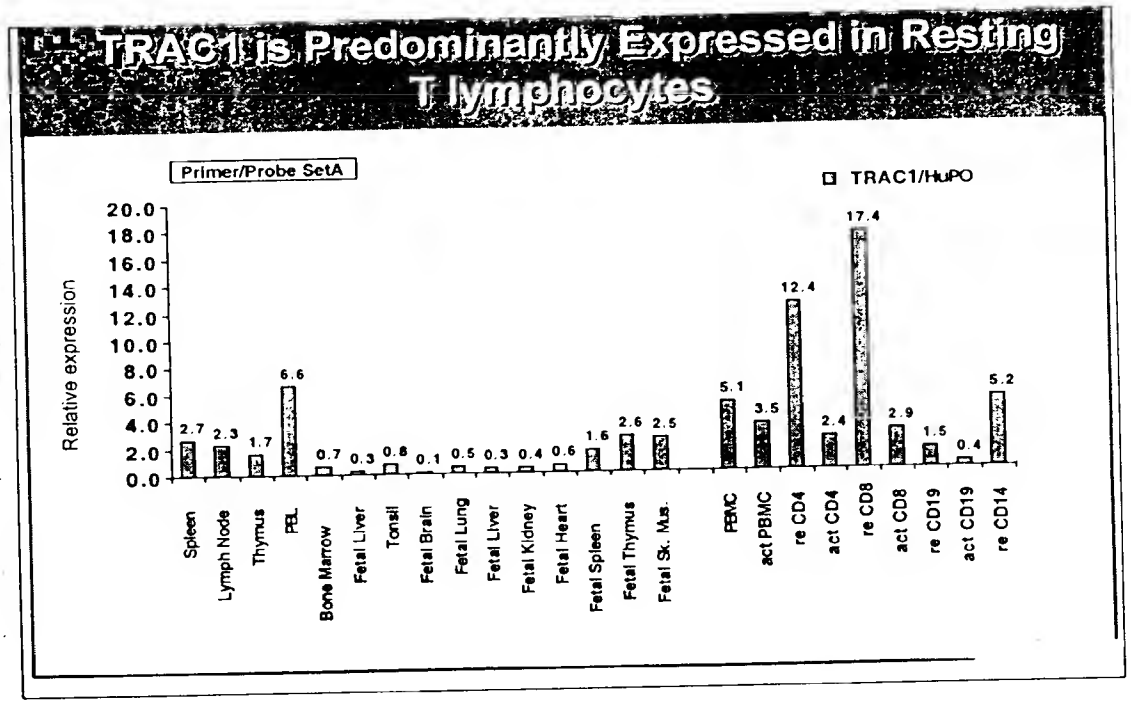


← FLJ20456

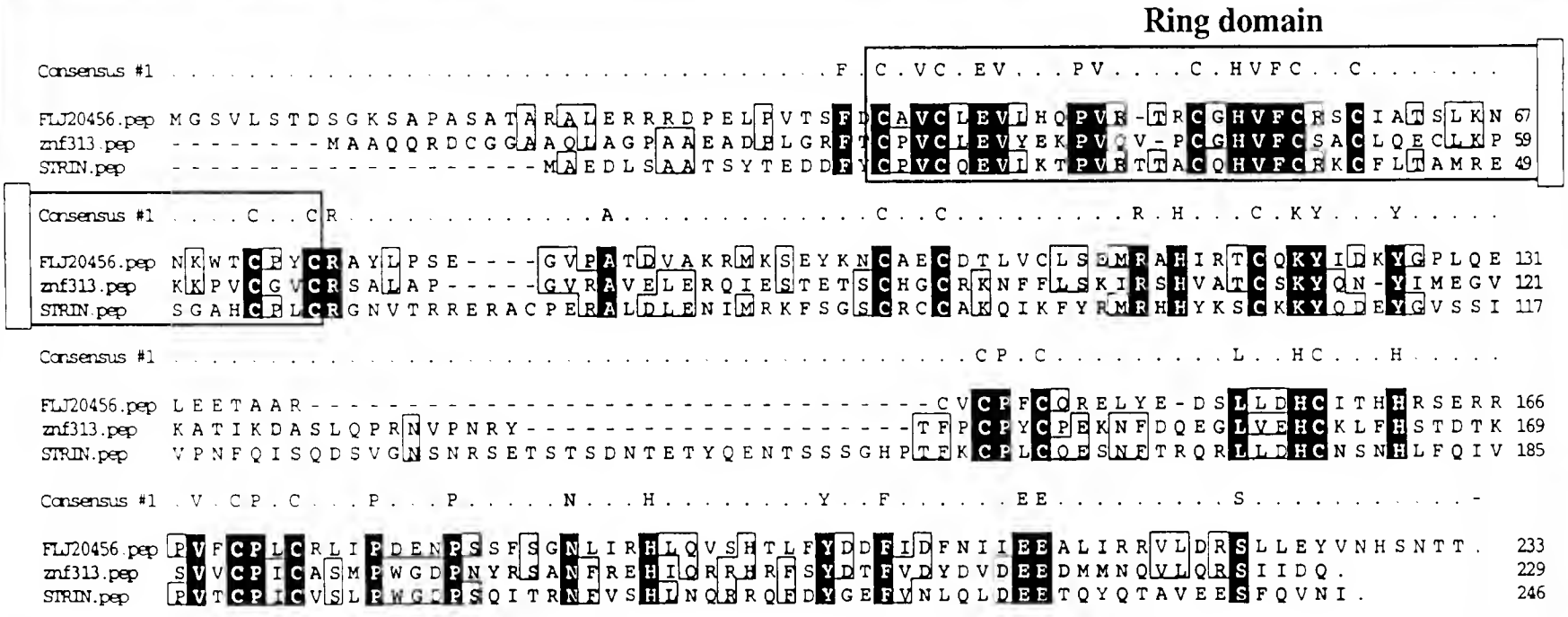
- FLJ20456 is expressed in multiple tissues
- The strongest expression is in PBL, Liver and Spleen

Figure 5

Figure 6


TRAC1 is Predominantly Expressed in Resting T lymphocytes
Primer/Probe SetA
TRAC1/HuPO
Relative expression
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
2.7
2.3
1.7
6.6
0.7
0.3
0.8
0.1
0.5
0.3
0.4
0.6
1.6
2.6
2.5
5.1
3.5
12.4
2.4
17.4
2.9
1.5
0.4
5.2
Spleen
Lymph Node
Thymus
PBL
Bone Marrow
Fetal Liver
Tonsil
Fetal Brain
Fetal Lung
Fetal Liver
Fetal Kidney
Fetal Heart
Fetal Spleen
Fetal Thymus
Fetal Sk. Mus.
PBMC
act PBMC
re CD4
act CD4
re CD8
act CD8
re CD19
act CD19
re CD14

FL20456 Sequence is Most Similar to Two Sequences: Znf313 and STRIN



- All three sequences are human
- Murine sequences are not shown

Percent Identity

divergence	1	2	3		
1		26.6	22.3	1	FLJ20456.pep
2	130.4		27.9	2	znf313.pep
3	140.9	134.7		3	STRIN.pep
	1	2	3		

Figure 7

Alignment of RING Domain Sequences from Various Human Proteins

```
Consensus #1  . . . . C . . C . . . . . . . . . . . . C . H . . C . . C . . . . . . . . . . . . . C . . . . . . . . . .

FLJ20456.Ring  V T S F D C A V C L E V L H Q P V R T R - C G H V F C R S C I A T S L - - K N N K W T C P Y C R A Y L P - S  50
znf313.Ring    L G R F T C P V C L E V Y E K P V Q V P - C G H V F C S A C L Q E C L - - K P K K P V C G V C R S A L A - P  50
STRIN.Ring     E D D F Y C P V C Q E V L K T P V R T T A C Q H V F C R K C F L T A M - - R E S G A H C P L C R G N V - - T  50
TRAF6.Ring     E S K Y E C P I C L M A L R E A V Q T P - C G H R F C K A C I I K S I - - R D A G H K C P V - D N E I L L E  50
c-Cbl.Ring     S T F Q L C K I C A E N - D K D V K I E P C G H L M C T S C L T S W - - Q E S E G Q G C P F C R C E I - K G  50
BRCA1.Ring     - - - L E C P I C L E L I K E P V S T K - C D H I F C K F C M L K L L N Q K K G P S Q C P L C K N D I T K R  50
BAR.Ring       V S E F S C H C C Y D I L V N P T - T L N C G H S F C R H C L A L W W A - S S K K T E C P E C R E K - - W E  50
RAG1.Ring      V K S I S C Q I C E H I L A D P V E T N - C K H V F C R V C I L R C L - - K V M G S Y C P S C R Y P C F - P  50
```

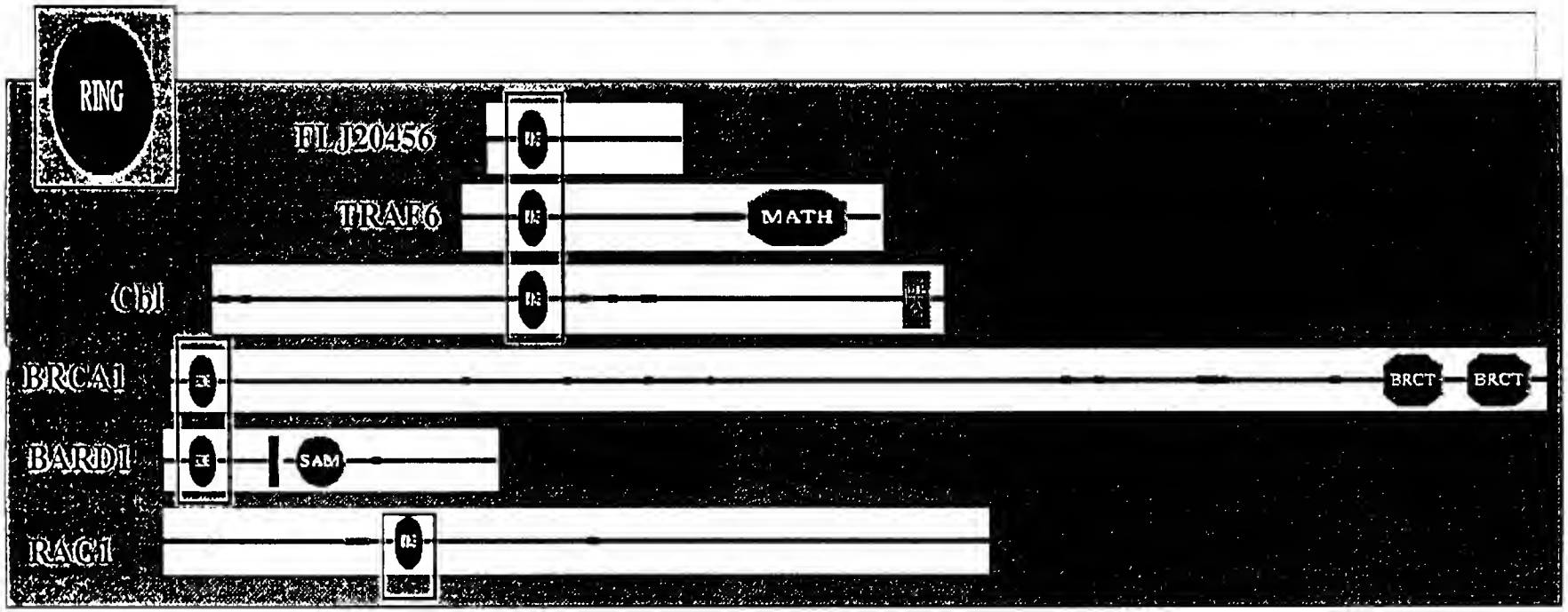


Figure 3

RING finger vs. Zinc finger proteins

Ring-HC: C_3HC_4 = Cys in position 5
Ring H2: C_3H2C_3 = His in position 5

- Ring finger domains have a conserved pattern of Cys and His residues that co-ordinate two zinc atoms to form a cross-brace structure
- Ring fingers are structurally distinct from zinc fingers

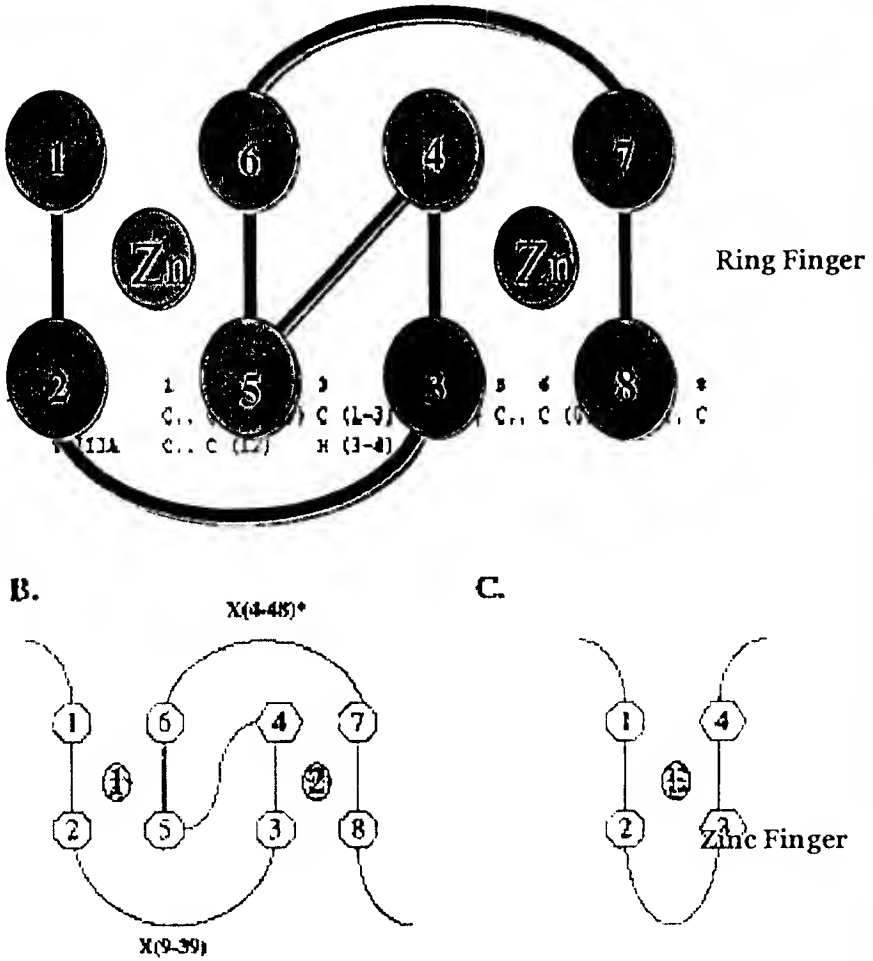


Figure 9

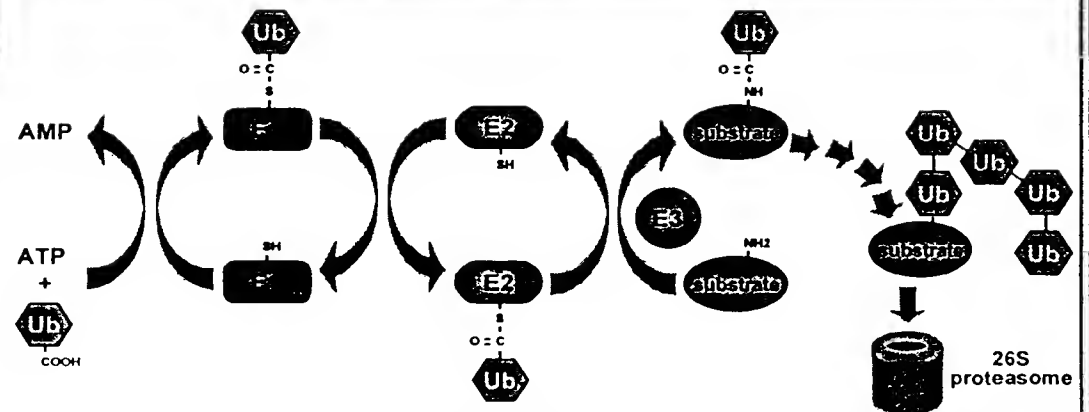
Figure 10

Ubiquitin Pathway Components

- E1: ubiquitin-activating enzyme, with a major isoform that may work broadly
- E2: ubiquitin-conjugating enzyme, a class of ~14 enzymes, interacts with E3
- E3: ubiquitin ligases, a broad and growing group of activities that promote addition of ubiquitin to specific proteins
- Proteasome-a 26S complex containing a 19S lid and base that mediates ATP- and ubiquitin-chain-dependent binding of substrates and a 20S catalytic core with three known proteolytic activities.

10A

Enzymology of Ubiquitynation

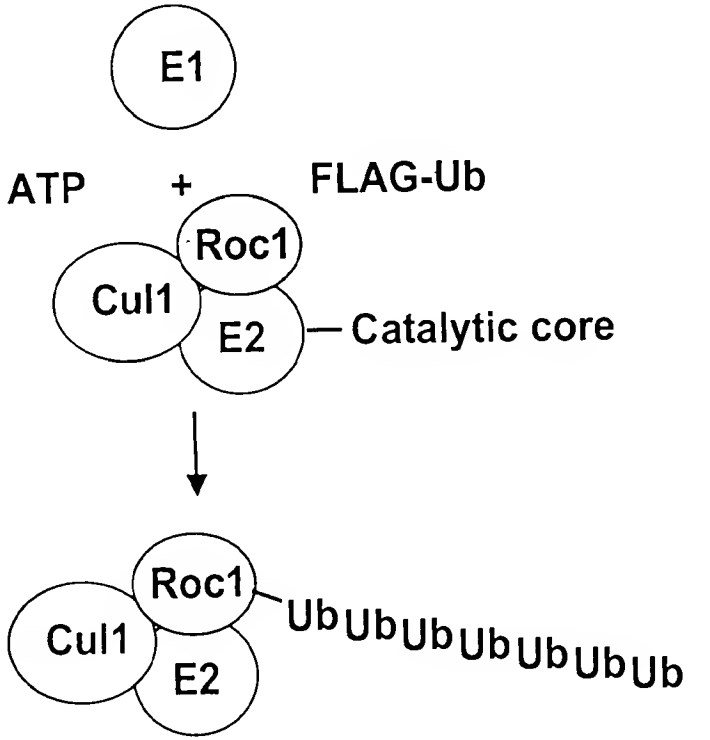


Ub = ubiquitin	E2 = ubiquitin conjugating enzyme
E1 = ubiquitin activating enzyme	E3 = ubiquitin ligase

10B

Figure 11a

A Reconstituted, Substrate-independent Assay for Studying Ligase Catalysis



The substrate-independent reaction has the same catalytic properties and requirements for Roc1/Cul1 as the substrate-dependent reaction

Reaction Components

E1:

E2 (UbcH5): GST-fusion (cleaved), E. coli

E3 (Ring/cullin): His-tagged, coexpressed, baculovirus

Ubiquitin: FLAG-tagged, E. coli

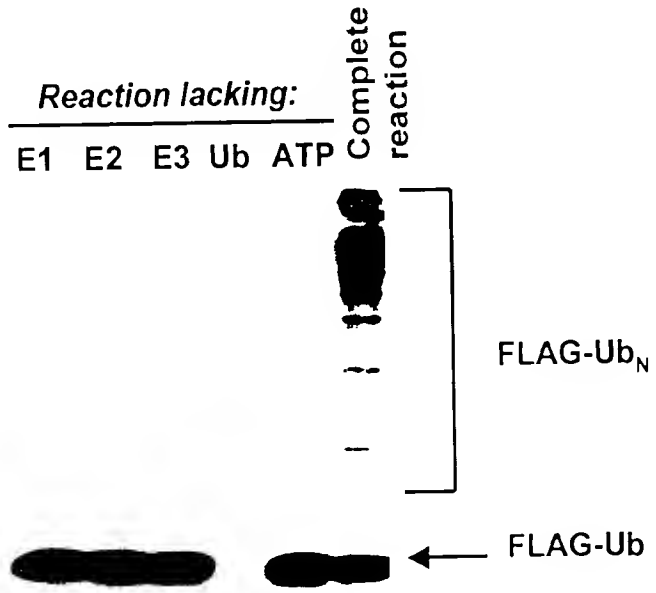


Figure 11 B

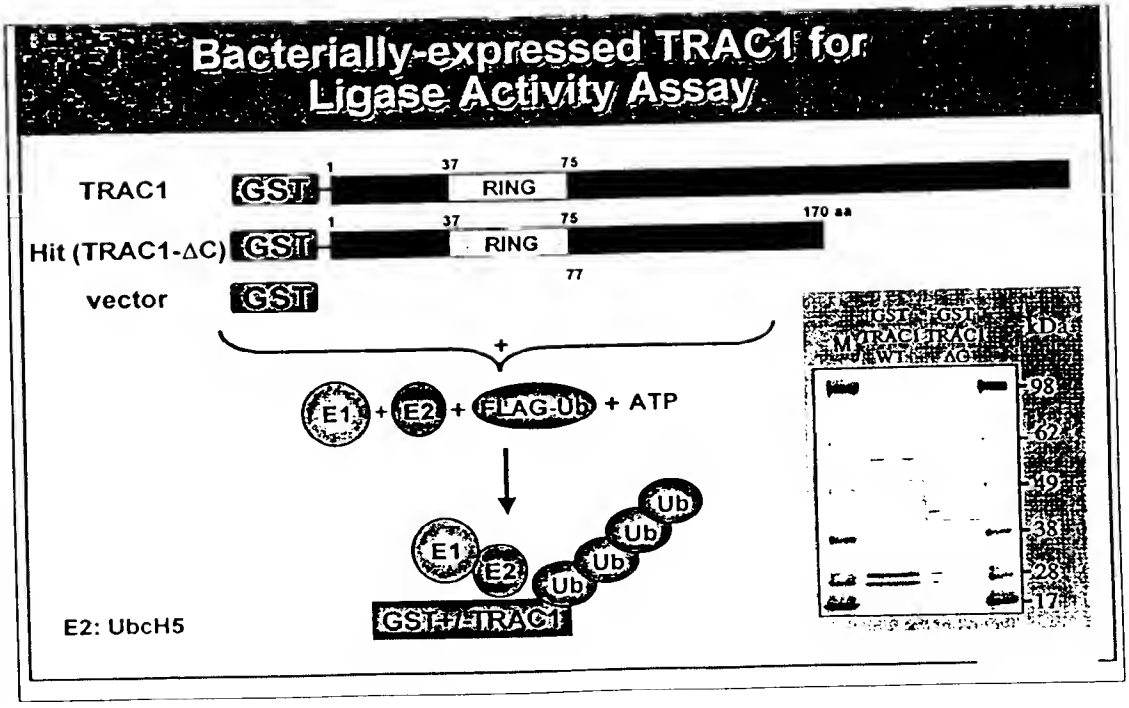
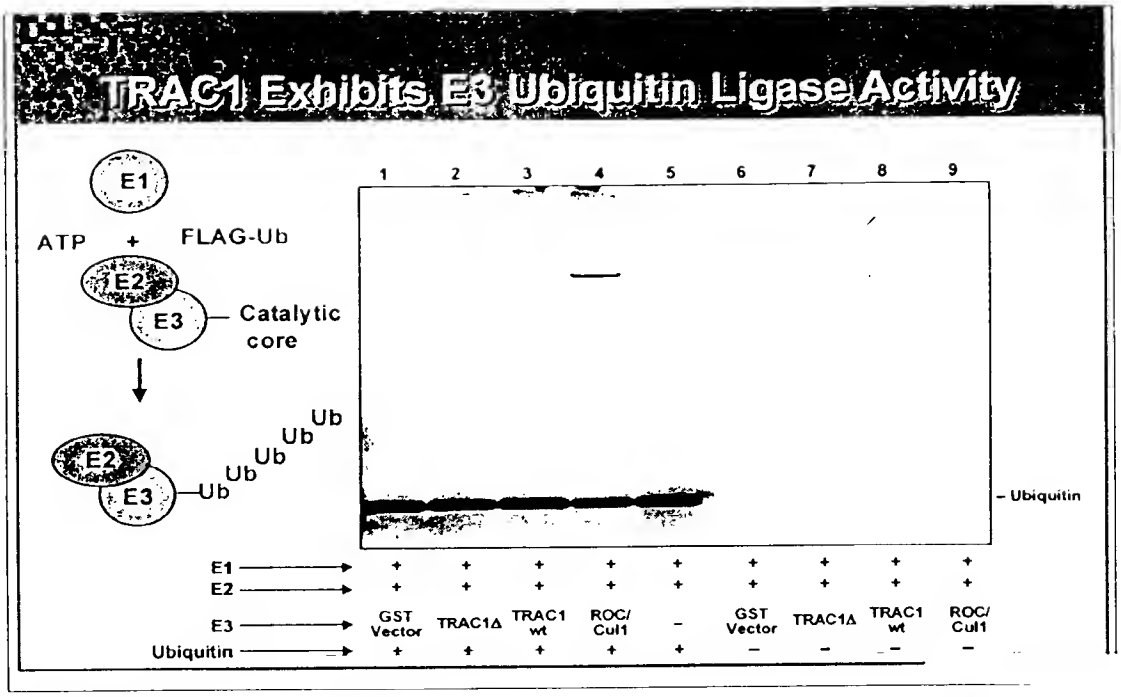

Bacterially-expressed TRAC1 for Ligase Activity Assay
TRAC1
GST
1
37
75
RING
Hit (TRAC1-ΔC)
170 aa
77
vector
+
E1
E2
FLAG-Ub
+ ATP
Ub
GST-hTRAC1
E2: UbcH5
kDa
98
62
49
38
28
17

Figure 12

12A



12B

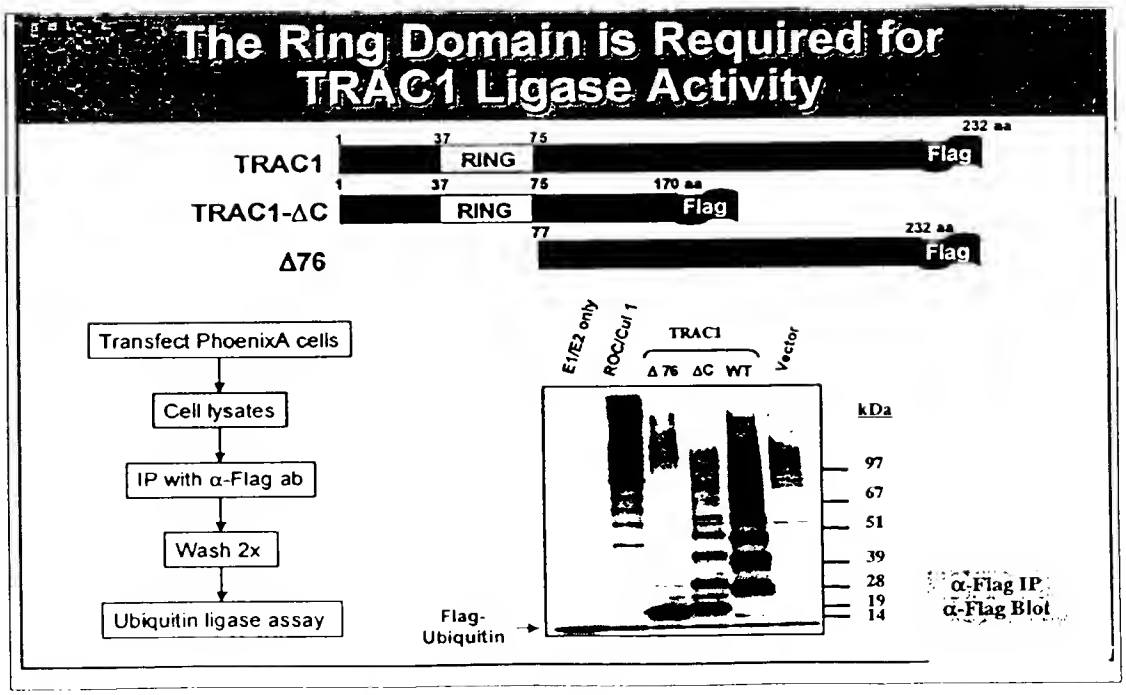
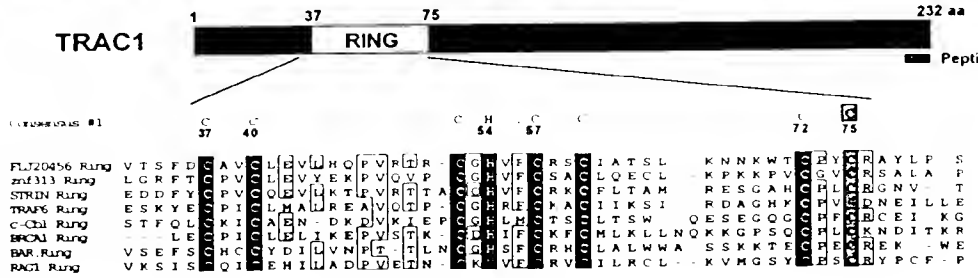


Figure 13

13A

Point mutations in Conserved Cysteine Residues of the TRAC1 Ring finger Domain



- The following expression plasmids were generated :
  pEFnig/Ring finger point mutants: H54A, C75A, C37,40A, H54C57A, C72,75A
  pEFnig/Myristylation site mutant: G2A

13B

Point mutations in Conserved Cysteine Resjdues of the TRAC1 Ring-finger Domain Disrupt Ligase Activity

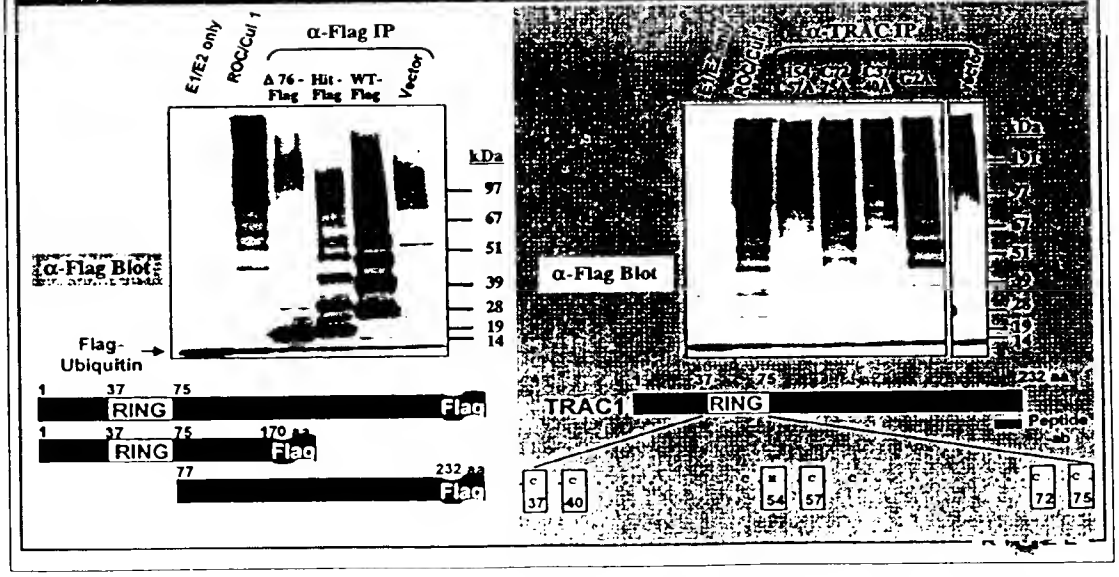


Figure 14

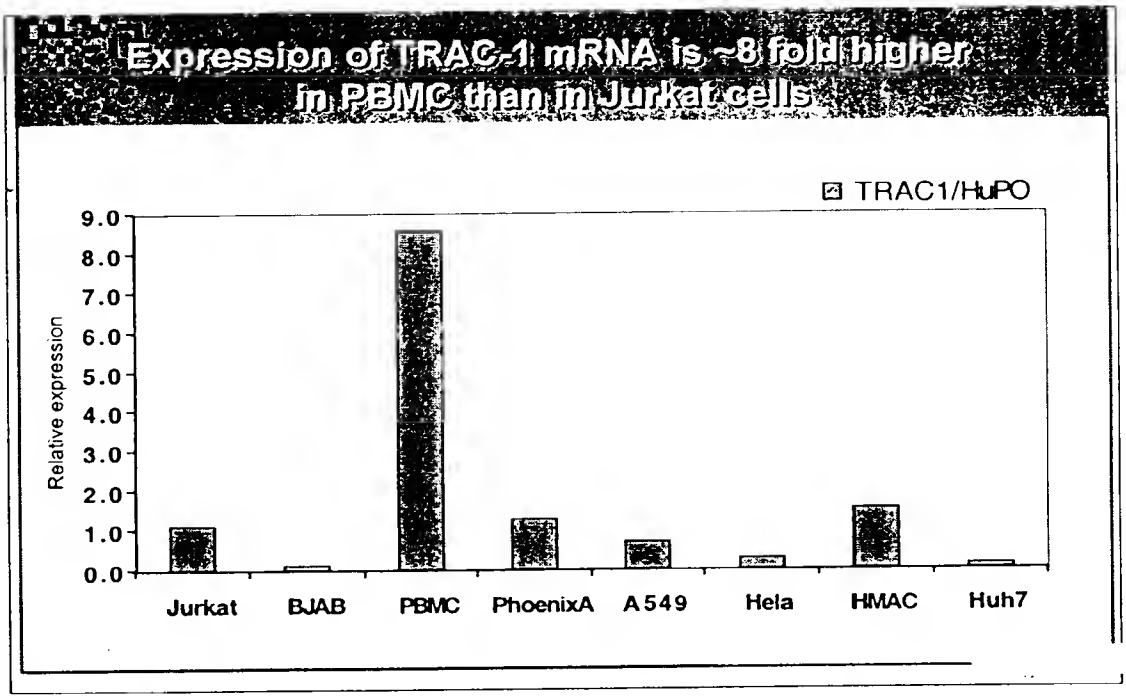

Expression of TRAC-1 mRNA is ~8 fold higher in PBMC than in Jurkat cells
TRAC1/HuPO
Relative expression
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Jurkat
BJAB
PBMC
PhoenixA
A549
Hela
HMAC
Huh7

Figure 15

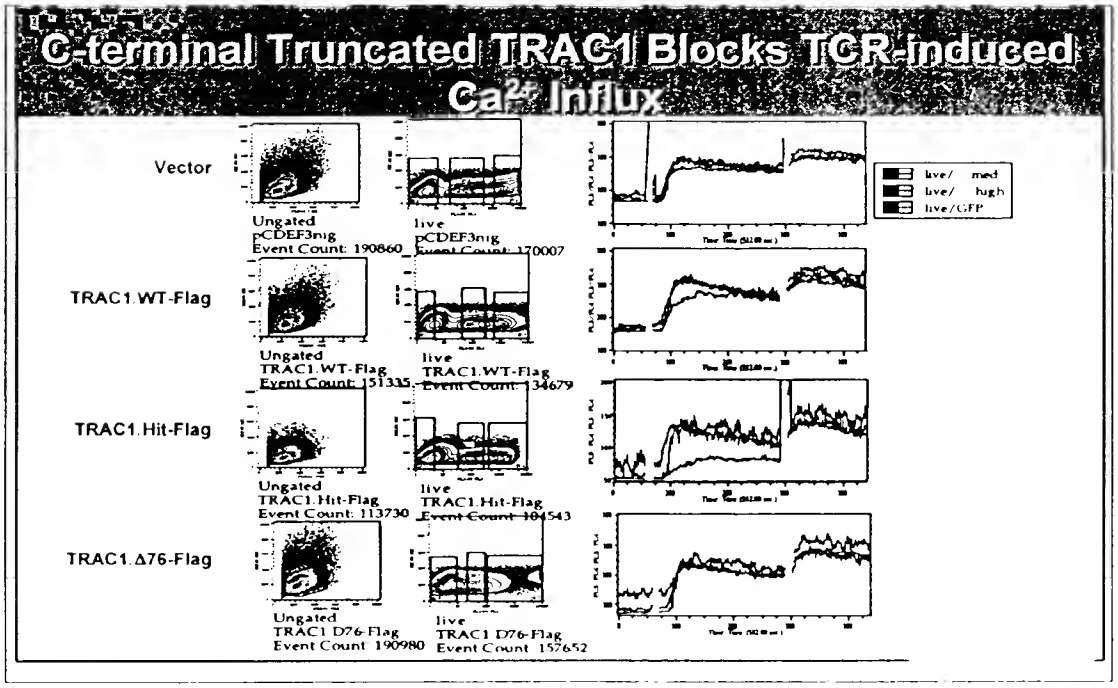

C-terminal Truncated TRAC1 Blocks TCR-induced Ca^{2+} Influx
Vector
TRAC1.WT-Flag
TRAC1.Hit-Flag
TRAC1.Δ76-Flag
Ungated
pCDEF3nig
Event Count: 190860
live
pCDEF3nig
Event Count: 170007
Ungated
TRAC1.WT-Flag
Event Count: 151335
live
TRAC1.WT-Flag
Event Count: 134679
Ungated
TRAC1.Hit-Flag
Event Count: 113730
live
TRAC1.Hit-Flag
Event Count: 104543
Ungated
TRAC1.D76-Flag
Event Count: 190980
live
TRAC1.D76-Flag
Event Count: 157652
live/ med
live/ high
live/GFP

Figure 16

An Intact TRAC1 Ring domain is Required for Inhibition of α–TCR-Induced CD69 Up-regulatio

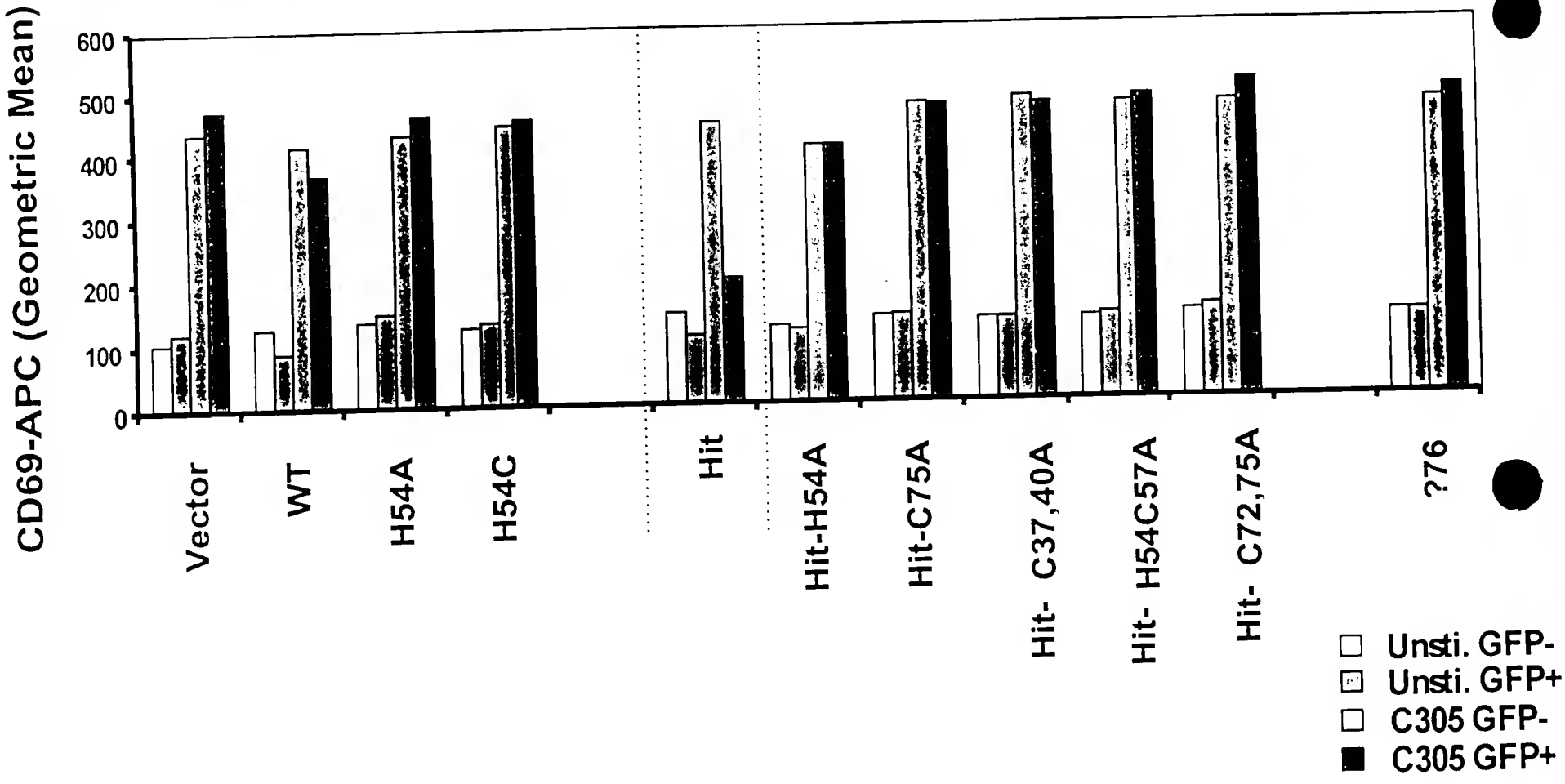
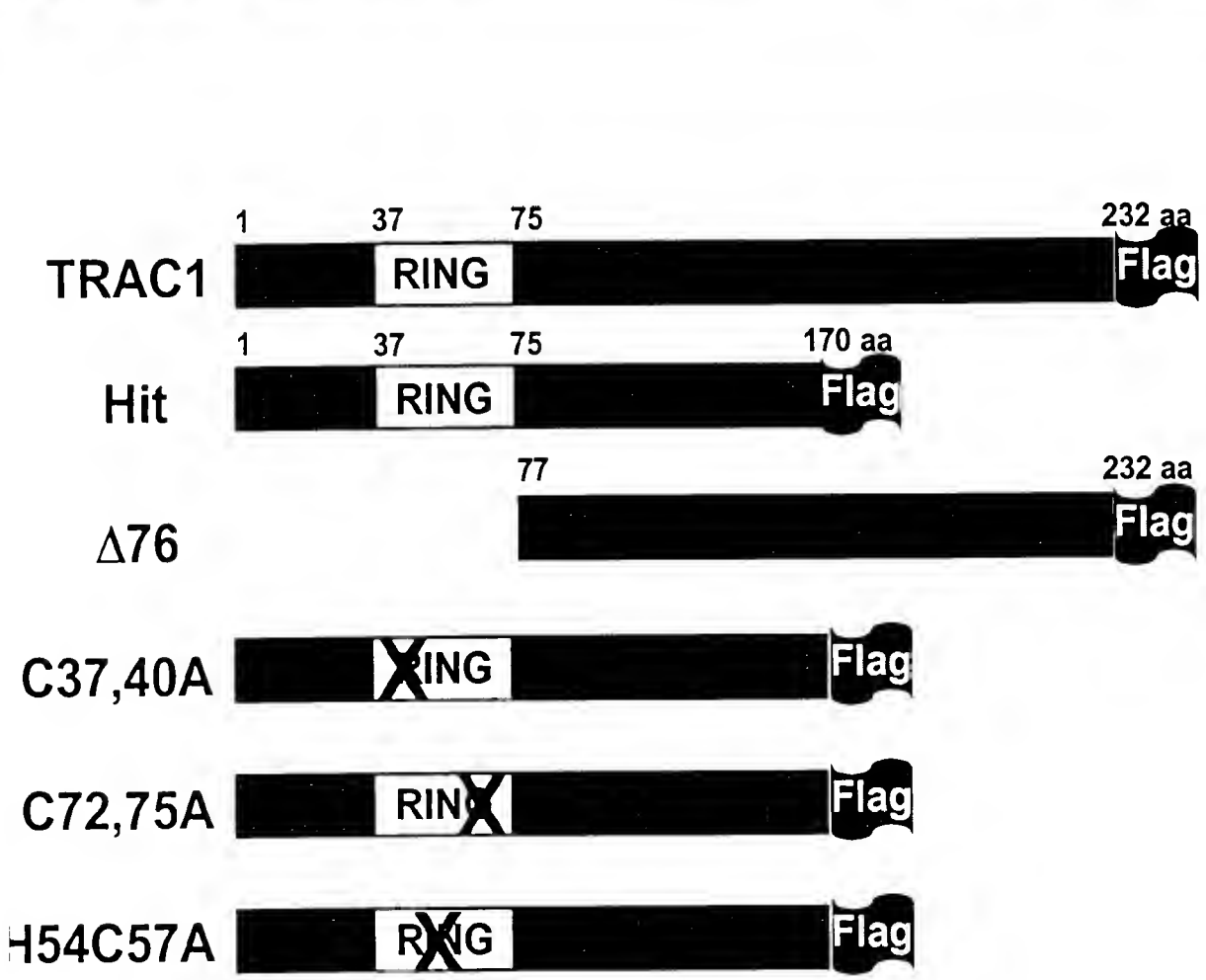


Figure 17

Summary of Functional Effects by Different TRAC-1 constructs



	Ubiquitin ligase activity	CD69 induction	Calcium mobilization
TRAC1	yes	-	+/-
Hit	yes	↓	↓
Δ76	no	-	-
C37,40A		-	-
C72,75A		-	-
H54C57A		-	-

Figure 18

Transiently Transfected TRAC1 Protein Binds to Ubiquitin-Conjugating Enzymes (E2s) UbcH7 and UbcH5 *in vitro*

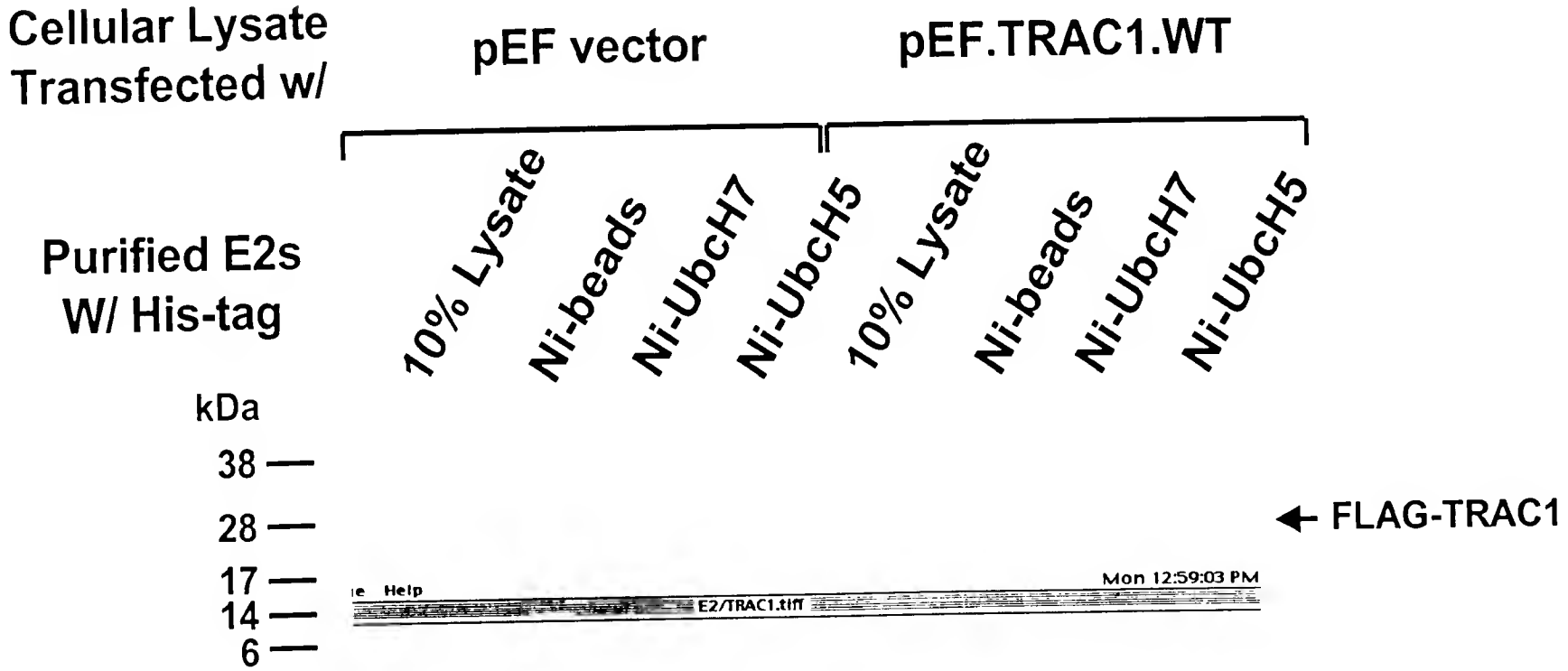
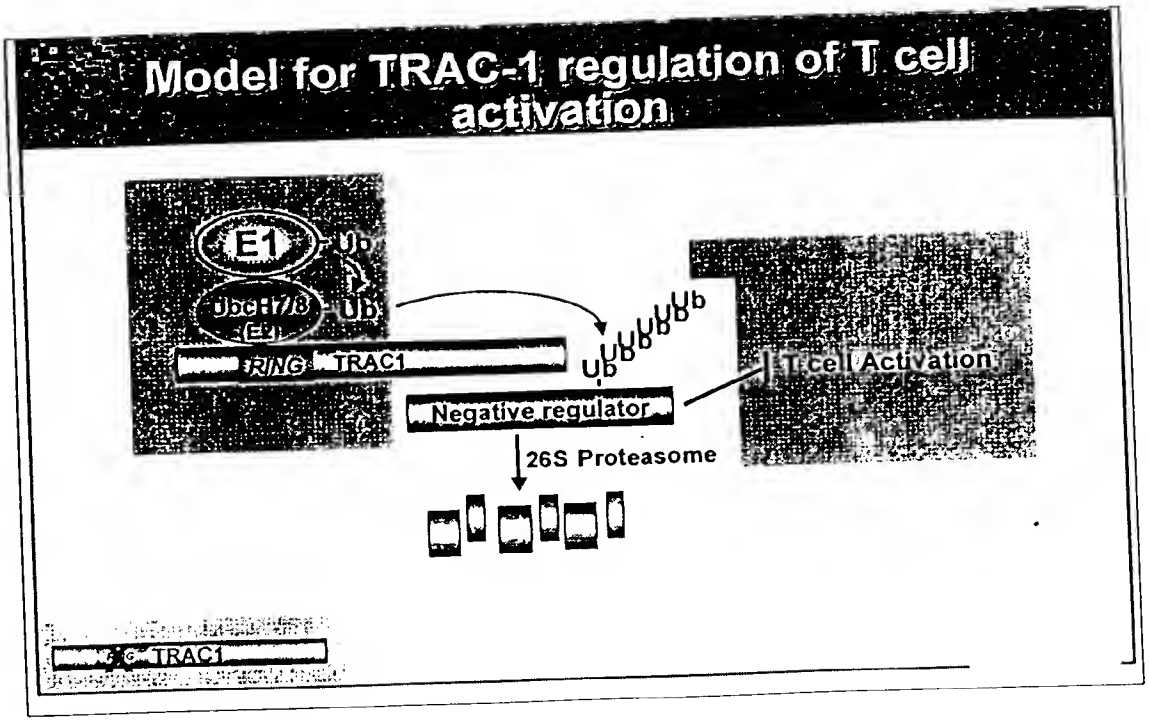


Figure 19


Model for TRAC-1 regulation of T cell activation
E1
Ub
Ubc H7/8 (E2)
Ub
RING
TRAC1
Ub
Negative regulator
26S Proteasome
T cell Activation
TRAC1